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Proline Utilization in *Saccharomyces cerevisiae*: Sequence, Regulation, and Mitochondrial Localization of the *PUT1* Gene Product

SY-SHI WANG AND MARJORIE C. BRANDRISS*

Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry of New Jersey-New Jersey Medical School, Newark, New Jersey 07103

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The *PUT1* gene of *Saccharomyces cerevisiae*, believed to encode proline oxidase, has been completely sequenced and contains an open reading frame capable of encoding a polypeptide of 476 amino acids in length. The amino terminus of the protein deduced from the DNA sequence has a characteristic mitochondrial import signal; two *PUT1-lacZ* gene fusions were constructed that produced mitochondrially localized β-galactosidase in vivo. The transcription initiation and termination sites of the *PUT1* mRNA were determined. By using a *PUT1-lacZ* gene fusion that makes a cytoplasmic β-galactosidase, the regulation of the *PUT1* gene was studied. *PUT1* is inducible by proline, responds only slightly to carbon catabolite repression, and is not regulated by the cytochrome activator proteins HAP1 and HAP2. The *PUT1* gene is under oxygen regulation; expression in anaerobically grown cells is 10-fold lower than in aerobically grown cells. Oxygen regulation is abolished when cells are respiratory deficient. *PUT1* expression in a [*rho*$^-$] strain grown either aerobically or anaerobically is as high as that seen in a [*rho*$^+$] strain grown aerobically. Studies on *PUT1* promoter deletions define a region between positions −458 and −293 from the translation initiation site that is important for full expression of the *PUT1* gene and required for oxygen regulation.

The conversion of proline to glutamate to provide *Saccharomyces cerevisiae* cells with a source of nitrogen takes place within mitochondria by the sequential action of two enzymes, proline oxidase and Δ^1-pyrroline-5-carboxylate (P5C) dehydrogenase. The enzymes are encoded by nuclear genes that are coregulated by proline induction and a control element encoded by the *PUT3* gene (6a, 8, 9). Mutations in the *PUT1* or *PUT2* genes result in deficiencies in proline oxidase or P5C dehydrogenase activities, respectively. Mutations in the *PUT3* gene result in either constitutive (9) or noninducible (6a) expression of these enzymes.

Previous work from this laboratory has focused on the regulation, DNA sequence, and subcellular localization of the second enzyme of the proline utilization pathway, P5C dehydrogenase, the product of the *PUT2* gene (6, 22). This enzyme is synthesized in the cytoplasm and imported into the matrix compartment of the mitochondria (7, 11). During the import process, a signal sequence of approximately 2 kilodaltons in length is removed from the amino terminus of the protein (J. Kaput and M. C. Brandriss, unpublished results).

In this report, we present studies on the regulation, sequence, and subcellular localization of the *PUT1* gene product. Although definitive proof is still lacking, there is mounting evidence that *PUT1* is the structural gene for proline oxidase, the first enzyme in the proline utilization pathway. *put1* mutants are defective in proline oxidase activity, and *put1*/+ heterozygotes show a gene dosage effect for enzyme activity (8, 9). The *PUT1* message is constitutively expressed in a strain carrying a regulatory mutation that causes constitutive proline oxidase activity (38). In an in vitro assay, the *PUT1* gene product is imported into yeast mitochondria and processed to its mature form (J. Kaput, S.-S. Wang, and M. C. Brandriss, unpublished results).

Proline oxidase has been studied in mammals, plants, fungi, and bacteria (3, 8, 12, 27). It is a mitochondrial or procaryotic plasma membrane-associated enzyme that requires a functional electron transport chain and aerobiosis for its activity. In *S. cerevisiae*, proline oxidase increases approximately 30-fold when proline is substituted for ammonia as the nitrogen source. Respiratory-deficient yeast strains contain inactive proline oxidase because of a nonfunctional electron transport chain, but in vitro activity can be detected if artificial electron acceptors are included in the assay (S.-S. Wang and M. C. Brandriss, unpublished results).

Because of the dependence of proline oxidase activity on respiration and aerobiosis, we were interested in extending our studies of the regulation of this enzyme to examine factors that affect the electron transport chain. With the cloned gene and the use of *PUT1-lacZ* gene fusions, we have tested and now report the effects of respiratory deficiency, carbon catabolite repression, anaerobiosis, and mutations in the cytochrome regulatory elements *HAP1* and *HAP2* on *PUT1* gene expression. In addition, we present the DNA sequence of the gene, its transcription initiation and termination sites, an analysis of the deduced amino acid sequence of the protein, and the mitochondrial localization of the gene product.

MATERIALS AND METHODS

Strains and media. Strains of *S. cerevisiae* used in this study are shown in Table 1. Strain C1500 is a respiratory-

* Corresponding author.

TABLE 1. Strains used in this study

Species and strain	Genotype	Reference or source
S. cerevisiae		
MB1000	*MAT*α wild type	Σ 1278b; J.-M. Wiame; (8)
MB1433	*MAT*α *trp1 ura3-52*	(6)
C1500	*MAT*α *trp1 ura3-52* [*rho*$^-$]	This study
LGW1	*MAT***a** *ade1-100 his4-519 leu2-3,112 ura3-52 hap2-1*	J. L. Pinkham
C70-1B	*MAT*α *his4-519 ura3-52 HAP2*	This study
C70-10A	*MAT***a** *his4-519 ura3-52 hap2*	This study
BWG1-7A	*MAT***a** *his4-519 leu2-3,112 ura3-52 ade1-100*	K. Pfeifer
WB32	*MAT***a** *his4-519 leu2-3,112 ura3-52 ade1-100 hap1-1*	K. Pfeifer
E. coli		
HB101	F$^-$ *hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44* λ$^-$	(4)
JM101	Δ(*lac-pro*) *supE thi* (F′ *traD36 lacI*^{q} *lacZ* Δ*M15*)	(39)

deficient derivative [*rho*$^-$] of strain MB1433 induced by ethidium bromide. Diploid C70 was constructed by crossing strain MB1433 to strain LGW1 (kindly provided by J. L. Pinkham). Strains C70-1B and C70-10A are meiotic products of diploid C70. Isogenic strains BWG1-7A and WB32 were kindly provided by K. Pfeifer. Media used for cell growth were described previously (8). Glucose (2%), galactose (0.5%), or raffinose (2%) was used as a carbon source. Nitrogen sources were either ammonium sulfate (0.2%, noninducing medium), ammonium plus proline (0.1%, partially inducing medium), or proline alone (fully inducing medium).

Genetic analysis. Mating, sporulation, and tetrad analysis were carried out by standard procedures (37).

Reagents. Restriction endonucleases and T4 polynucleotide kinase were purchased from New England BioLabs, Inc. or Boehringer Mannheim Biochemicals and were used in accordance with the instructions of the manufacturers. Avian myeloblastosis virus reverse transcriptase was purchased from Life Sciences, Inc. Calf intestinal phosphatase was purchased from Sigma Chemical Co. S1 nuclease was obtained from Miles Laboratories, Inc. Radionucleotides were purchased from Amersham Corp. Oligodeoxythymidylic acid-cellulose was obtained from Collaborative Research, Inc.

RNA and DNA preparation and DNA transformation of *Escherichia coli* and *S. cerevisiae*. Methods for RNA and DNA preparation and DNA transformation of *E. coli* and *S. cerevisiae* have been described previously (38).

DNA sequencing. Restriction fragments of a 3-kilobase (kb) *Kpn*I fragment carrying the *PUT1* gene were cloned into M13 vectors mp18 and mp19 (39). The sequencing was carried out by the dideoxynucleotide chain termination method of Sanger et al. (35). The primer used was complementary to 17 base pairs (bp) at a region 40 nucleotides from the polylinker.

mRNA mapping. The precise location of the 5′ end of the *PUT1* mRNA was determined by using the primer extension technique (24) in conjunction with dideoxy sequencing. The poly(A)$^+$ RNAs were prepared from wild-type strain MB1000 grown in an inducing (0.1% proline) medium. The 3-kb *Kpn*I DNA fragment from plasmid pWB8 was cloned into bacteriophage mp18, and the single-stranded template (corresponding to the mRNA strand) was used for the sequencing ladders. The 62-bp *Msp*I-*Ava*II fragment (see Fig. 4A) was dephosphorylated with calf intestinal phosphatase, and the 5′ end of this fragment was labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. The labeled DNA was first heat-denatured for 10 min and then hybridized with or without 5 μg of poly(A)$^+$ RNA at 85°C for another 10 min. The DNA-RNA mixture was transferred to a 50°C bath and incubated for 15 to 18 h. The DNA was extended along the hybridized RNA with reverse transcriptase, and the resulting product was subjected to electrophoresis on a sequencing gel. Sequencing reactions, with the same primer and the 3-kb DNA template, were carried out and electrophoresed on the same gel.

The 3′ end of the *PUT1* transcript was mapped by the S1 mapping technique of Berk and Sharp (2). A 500-bp *Bgl*II-*Kpn*I fragment (see Fig. 4B) was prepared and was 3′ end labeled by filling in the *Bgl*II restriction site with reverse transcriptase (25). The labeled DNA was mixed with or without 5 μg of poly(A)$^+$ RNA and placed at 70°C for 10 min. The mixture was immediately transferred to a 48°C bath for 15 to 18 h. The hybrids were then digested with S1 nuclease at an empirically determined nuclease concentration (0.9 U/μl) and digestion temperature (45°C). The protected labeled DNA was resolved on a sequencing gel together with the sequencing ladder by using a 59-bp *Bgl*II-*Stu*I DNA fragment as the primer and the single-stranded 3-kb *Kpn*I DNA as the template (see Fig. 4B).

Construction of the *PUT1-lacZ* fusions. Three in-frame *PUT1-lacZ* fusion plasmids that contain the 1-kb upstream region and the initial 25, 128, or 455 codons of the *PUT1* open reading frame, fused to the coding region of the *lacZ* gene, were constructed by ligation of convenient restriction fragments. The number following *lacZ* indicates the number of codons of the *PUT1* gene contained in the gene fusion.

To construct the *PUT1-lacZ25* plasmid, a *Cla*I-*Pst*I *PUT1* DNA fragment was isolated from plasmid pWB8 (38) and cloned into M13 vector mp18 digested with *Acc*I and *Pst*I to generate clone AP18 (see Fig. 1). Clone AP18 was digested with endonuclease *Ava*II (at codon 25 of *PUT1*), and the ends were filled in by using reverse transcriptase, followed by digestion with *Eco*RI. Plasmid E356 (kindly provided by A. Myers [30]) is a 2-μm *lacZ* expression vector with a polylinker site located in front of the *lacZ* gene. To construct an in-frame *PUT1-lacZ* fusion, vector E356 was digested with *Sal*I and the ends were filled in, and it was then digested with *Eco*RI. The *Eco*RI-*Ava*II DNA fragment from clone AP18 was ligated to the *Eco*RI-*Sal*I E356 vector to form plasmid pWB35.

To construct a *CEN* plasmid carrying the *PUT1-lacZ* fusion, plasmid pWB35 was digested with *Eco*RI and *Stu*I

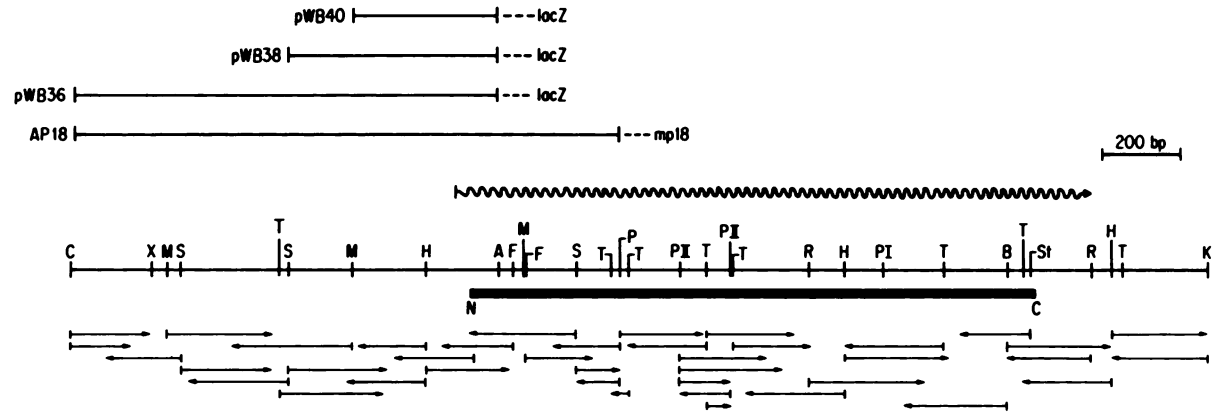


FIG. 1. DNA sequencing strategy for the *PUT1* gene. A restriction map of the 2.9-kb *Cla*I-*Kpn*I fragment of plasmid pWB8 is shown. Symbols: ⊢→ and ←⊣, length and direction of sequences determined from M13 clones; ▬, open reading frame of the *PUT1* polypeptide with amino (N) and carboxy (C) termini indicated; ⟿, position and direction of the *PUT1* mRNA. Clone AP18 contains the *Cla*I-*Pst*I *PUT1* DNA fragment in the phage vector M13mp18. Plasmids pWB36, pWB38, and pWB40 are *PUT1-lacZ* fusion plasmids with various lengths of the *PUT1* upstream sequence ligated to the *Ava*II site at codon 25 of the *PUT1* open reading frame (see Materials and Methods). Abbreviations: A, *Ava*II; B, *Bgl*II; C, *Cla*I; F, *Fnu*4HI; H, *Hin*dIII; K, *Kpn*I; M, *Msp*I; P, *Pst*I; PI, *Pvu*I; PII, *Pvu*II; R, *Eco*RV; S, *Sau*3A; St, *Stu*I; T, *Tag*I; X, *Xho*I. Additional *Fnu*4HI and *Sau*3A sites are not shown in this map.

and a 4.8-kb DNA fragment containing the in-frame *PUT1-lacZ25* gene fusion was isolated and ligated to plasmid YCp50 digested with *Eco*RI and *Nru*I, forming plasmid pWB36 (Fig. 1). The junctions of the *PUT1-lacZ25* gene fusion were confirmed by DNA sequencing.

Fusion plasmids *PUT1-lacZ128* and *PUT1-lacZ455* were constructed and placed in plasmid YCp50 in a similar two-step procedure as described above, except *PUT1-lacZ128* was constructed by using a *Pst*I site at codon 128 and *PUT1-lacZ455* was constructed by using a *Bgl*II site at codon 455 in the open reading frame of the *PUT1* gene.

Deletion formation in the *PUT1* upstream region. A 539-bp *Sau*3A-*Pst*I fragment from plasmid pWB35 that carried promoter sequence −458 to −1 and DNA through codon 25 of *PUT1* was ligated back to plasmid pWB35 cut with *Bam*HI and *Pst*I. This plasmid, pWB37, was deleted for the upstream promoter sequence −1010 to −458. This *PUT1-lacZ25* DNA was moved to the *CEN* plasmid YCp50 by the methods described above, and the resulting plasmid was called pWB38 (Fig. 1).

A 374-bp *Msp*I-*Pst*I fragment from plasmid pWB35 that carried promoter sequence −293 to −1 and DNA through codon 25 of *PUT1* was isolated, and the *Msp*I end was blunted with reverse transcriptase. This fragment was ligated back to plasmid pWB35 cut with *Sma*I and *Pst*I to form plasmid pWB39. The *CEN* plasmid carrying the *PUT1-lacZ25* gene fusion with this promoter deletion (−1010 to −294) was called pWB40 (Fig. 1).

Growth of *S. cerevisiae* for enzyme assays and preparation of cell extracts. The methods for growth of aerobic cultures have been described (7).

For anaerobic growth, cells were inoculated lightly (5×10^3 cells per ml) into media containing 2% glucose and 0.2% ammonium sulfate or ammonium sulfate plus 0.1% proline, supplemented with ergosterol (20 μg/ml), Tween 80 (0.2%), and amino acids, as required. Cells were grown anaerobically for 10 to 12 generations (about 4 days) in flasks packed into a sealed jar containing a GasPak Anaerobic System (BBL Microbiology Systems). The cells were harvested in mid-log phase at a density of 2×10^7 cells per ml. To compare the β-galactosidase activities of aerobically grown cells to anaerobically grown cells, the media for aerobic growth were also supplemented with ergosterol and Tween 80. Methods for preparation of extracts have been described (7).

Cellular fractionation and enzyme assays. Yeast cells were fractionated into cytosolic and crude mitochondrial fractions by the method of Daum et al. (14). Briefly, yeast cells were grown in a medium containing 0.5% galactose, 0.1% ammonia, 0.1% proline, and tryptophan (20 mg/liter) and harvested late in logarithmic phase. Spheroplasts were prepared and then lysed with a Dounce homogenizer. The cell lysate was centrifuged at 1,500 × *g* for 5 min (E. I. DuPont De Nemours & Co., Inc.). The supernatant was collected and subjected to further centrifugation at 10,000 × *g* for 10 min. The supernatant from this high-speed centrifugation contained the cytosolic components, and the pellet contained most of the mitochondria.

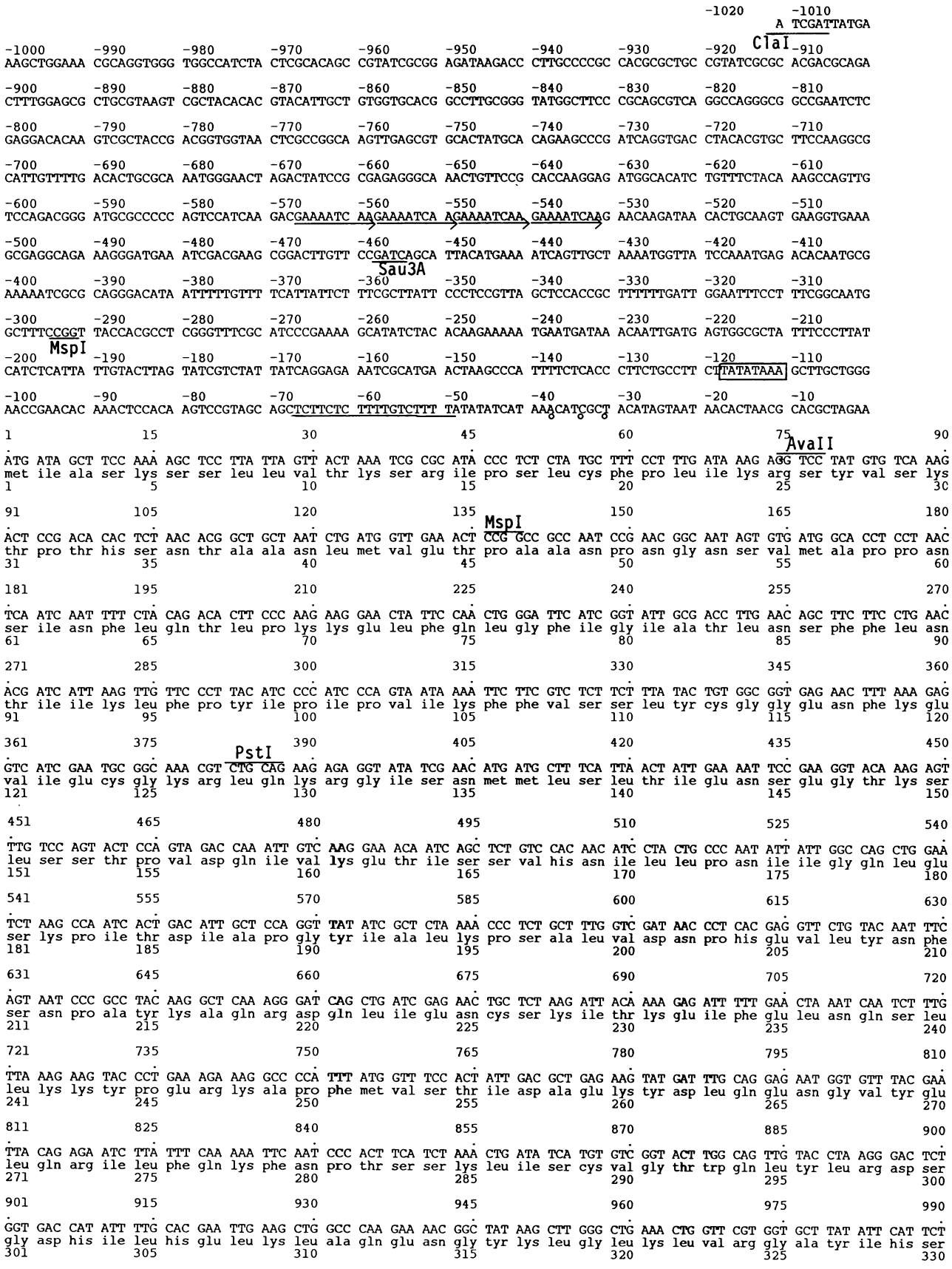
The activities of the enzymes glucose-6-phosphate dehydrogenase (13), cytochrome *c* oxidase (26), fumarase (34), and β-galactosidase (29) in each fraction were determined as described. Protein concentration was determined by the method of Bradford (5) with crystalline bovine serum albumin as the standard.

RESULTS

Nucleotide sequence of the *PUT1* gene. We previously reported the cloning and characterization of the *PUT1* gene of *S. cerevisiae* carried on a 3-kb *Kpn*I restriction fragment in plasmid pWB8 (38). To determine the deduced amino acid sequence of the *PUT1* gene product and compare the upstream regulatory sequence to that of the coregulated *PUT2* gene, we sequenced the entire 3-kb *PUT1* DNA. The sequencing strategy is outlined in Fig. 1. The sequence of 2,881 nucleotides containing the *PUT1* gene and its 5′ and 3′ flanking regions is shown in Fig. 2.

The sequence analysis revealed a long open reading frame from nucleotides +1 to +1428. The first ATG is surrounded by purines at positions −3 (G) and +4 (A), bases that were proposed to play a role in translation (21). A TATATAAA sequence is located at position −118 with respect to the initiation of translation of the open reading frame.

Analysis of the 440-bp DNA downstream of the *PUT1* reading frame indicates that it possesses an A+T content of


ClaI
Sau3A
MspI
MspI
AvaII
PstI

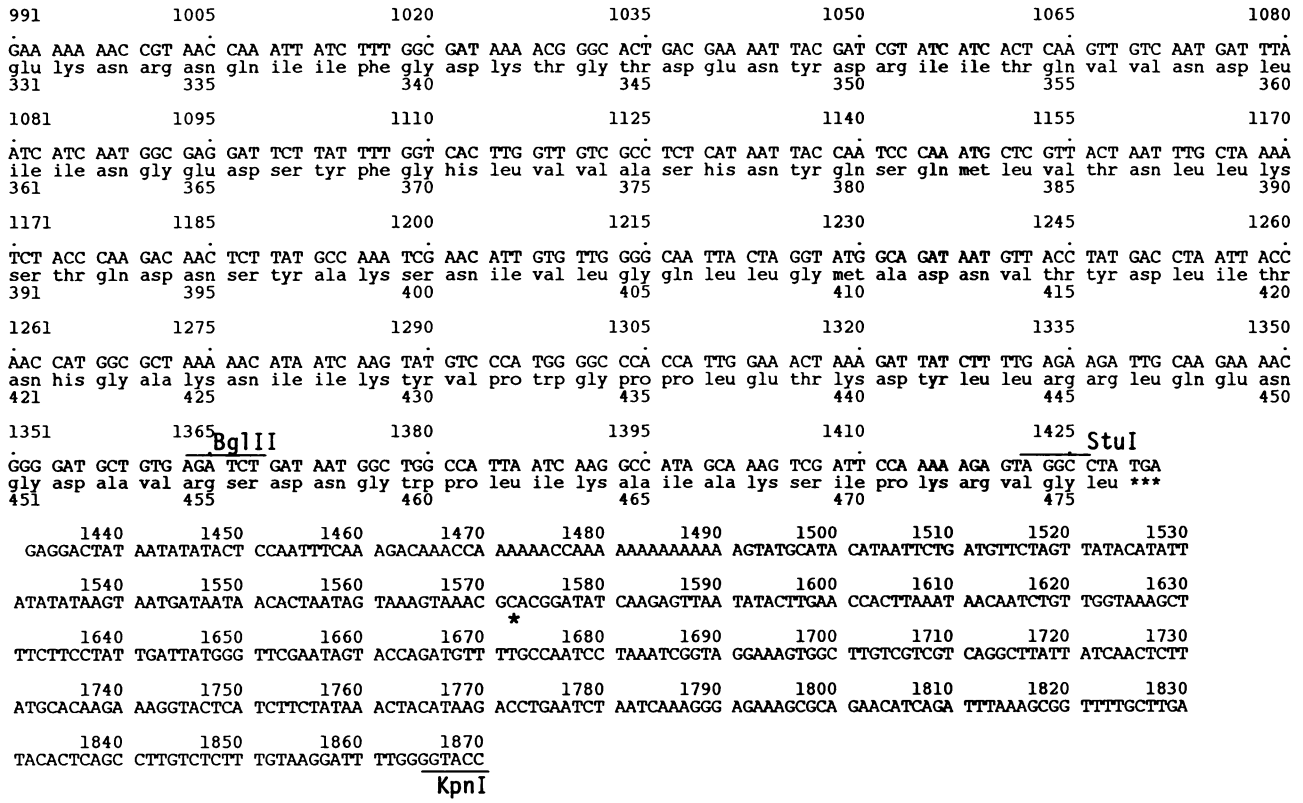


FIG. 2. *PUT1* DNA and deduced amino acid sequences. The putative *PUT1* protein-coding region extends from nucleotides 1 to 1428. The major transcription start sites and the termination site are indicated by open circles and an asterisk, respectively, under the sequence. The TATA sequence is boxed, and the pyrimidine-rich region is underlined. The 9-bp tandem repeat is indicated by arrows under the sequence. Specific restriction sites referred to in the text are indicated.

67% and multiple translational stop codons in all three reading frames. No putative polyadenylation signal, AATAAA (16), tripartite terminator (TAG. . .TAGT . . .TTT), or TTTTTATA sequence (18, 40) was found in this region.

An unusual 9-bp sequence, GAAAATCAA, perfectly repeated four times in tandem, was found to be located at positions −567 to −532. Deletion analysis (see below) suggested that the region containing this repeat was not required for the expression and regulation of the *PUT1* gene.

Deduced amino acid sequence of the *PUT1* gene product. The predicted sequence of the 476-amino acid polypeptide encoded by the *PUT1* gene is also shown in Fig. 2. The choice of the initiating methionine was substantiated by the behavior of *PUT1-lacZ* gene fusions (see below). The predicted molecular weight of this protein was 53,418. The amino terminus has features characteristic of mitochondrially imported proteins in that within the first 43 residues, there are 7 positively charged amino acids, no negatively charged residues, and 12 hydroxylated amino acids. Codon usage analysis of the *PUT1* protein (data not shown) indicates that there is very little codon bias, suggestive of a moderately expressed gene (1, 20).

On the basis of the hydropathy analysis of the deduced amino acid sequence (Fig. 3), the *PUT1* protein contains a hydrophobic region from residues 75 to 112 that is long enough to span a membrane (31).

Fine-structure mapping of the *PUT1* transcripts. The positions of the 5′ ends of the *PUT1* transcripts were mapped by the primer extension method (24) as described in Materials and Methods. The result of this 5′-end mapping is shown in Fig. 4A. The major transcription initiation sites were located at positions −31, −34, and −38 with respect to the translation initiation site. Several minor start sites occurred between positions −15 and −44.

The 3′ terminus of the *PUT1* mRNA was localized by the S1 nuclease protection method as described in Materials and Methods. As shown in Fig. 4B, there is a single termination site on the *PUT1* DNA that corresponds to a location 141 nucleotides 3′ of the stop codon.

Subcellular location of the *PUT1* gene product. To determine the cellular location of the *PUT1* gene product, three *PUT1-lacZ* fusion plasmids were constructed. These gene fusions contained the entire *PUT1* promoter and the initial 25, 128, or 455 codons of the open reading frame fused to the coding region of the *lacZ* gene and were placed on low-copy-number (*CEN*) plasmids. The plasmids were introduced into yeast strain MB1433, and the transformants were isolated and analyzed. Strains harboring the *PUT1-lacZ455* and the *PUT1-lacZ128* gene fusions grew poorly on a medium that contained galactose, ammonia, and proline, with doubling times of 10 and 5 h, respectively. The doubling time of the strain carrying the shortest fusion *PUT1-lacZ25* grown under the same conditions was similar to that of the wild-type strain lacking a gene fusion (2.5 h).

To determine the cellular location of these *PUT1-lacZ* hybrid proteins, cell fractionation experiments were conducted as described in Materials and Methods. Yeast cells

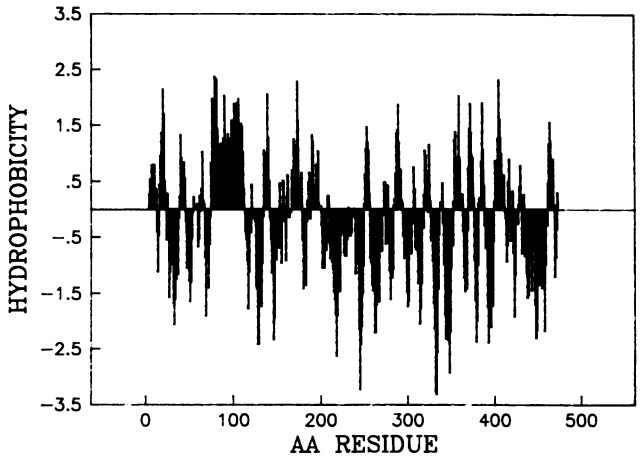


FIG. 3. Hydropathy plot of predicted *PUT1* protein. The plot of the hydropathy values was generated by averaging seven residues and plotting the average over the fourth residue according to the method of Kyte and Doolittle (23). Positive values indicate hydrophobicity, and negative values indicate hydrophilicity.

carrying the fusion plasmids were grown on a partially inducing (ammonium plus proline) medium until late in log phase and fractionated into cytoplasmic and mitochondrial fractions. The β-galactosidase activity in each fraction was measured, and the distribution of the enzyme activity was compared to that of marker enzymes. Glucose-6-phosphate dehydrogenase served as a marker of the cytoplasmic fraction, and cytochrome *c* oxidase was used as a marker of the mitochondrial fraction.

The activities and distribution of the enzymes found in these fractions are shown in Table 2. Glucose-6-phosphate dehydrogenase was found predominantly in the cytoplasmic fraction, whereas cytochrome *c* oxidase was found to be associated with the mitochondria. The *PUT1-lacZ128* and *PUT1-lacZ455* hybrid proteins behaved in a manner similar to cytochrome *c* oxidase with 94 and 90% of activities, respectively, in the mitochondrial fraction. On the other hand, the shortest *PUT1-lacZ25* hybrid protein was found predominantly in the cytoplasm and behaved identically to a cytoplasmically localized *CYC1-lacZ* fusion protein (data not shown).

Effectors of *PUT1* gene expression. The regulation of *PUT1* gene expression was studied by measuring the effects of

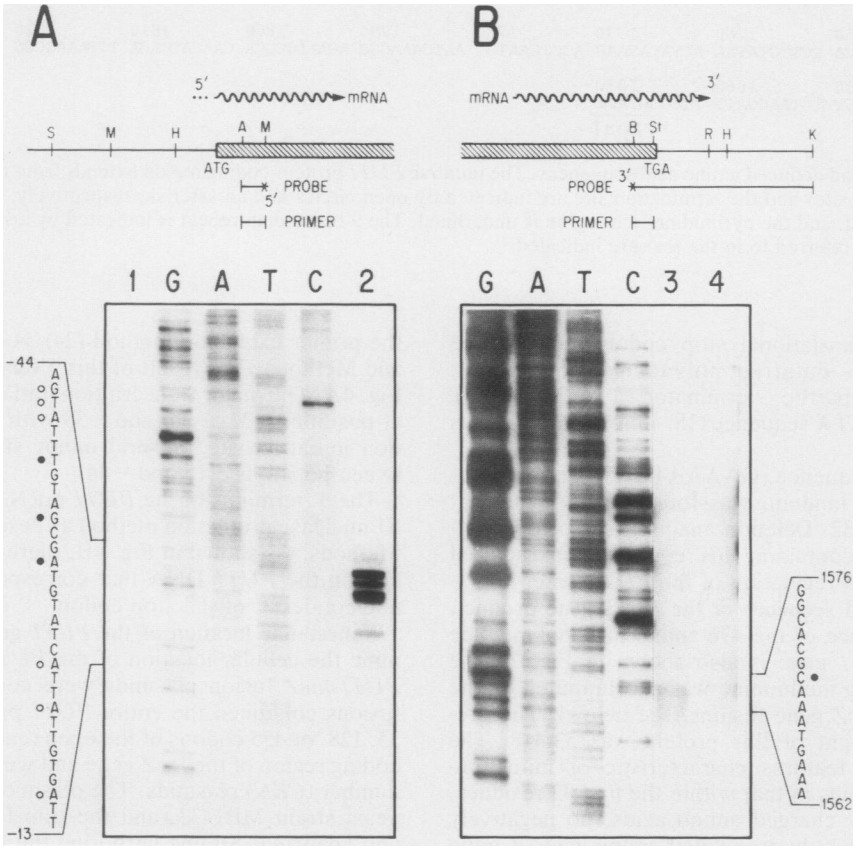


FIG. 4. Mapping of the 5′ and 3′ ends of the *PUT1* transcripts. (A) Primer extension analysis of the 5′ termini. The primer used was a 62-bp *Ava*II-*Msp*I fragment with a labeled 5′ end. The hybridization conditions were as described in Materials and Methods. The primer was extended with reverse transcriptase, and the extended products (lanes 1 and 2) were resolved on an 8% acrylamide sequencing gel. RNA was omitted in the reaction shown in lane 1. The sequence of the noncoding strand was determined (lanes G, A, T, and C) with the same 62-bp fragment as the primer and is shown on the left. Symbols: ● and ○, major and minor transcription starts, respectively. (B) S1 mapping of the 3′ terminus of the *PUT1* mRNA. A 500-bp *Bgl*II-*Kpn*I fragment was labeled at the 3′ end and used as the probe. Hybridization and S1 conditions were as described in Materials and Methods. Protected DNA fragments (lanes 3 and 4) were resolved on an 8% acrylamide sequencing gel. Yeast poly(A)$^+$ RNA was omitted in the reaction shown in lane 4. By using the 59-bp *Bgl*II-*Stu*I fragment as a primer, the sequence of this region was determined (lanes G, A, T, and C) and is shown on the right. The filled-in circle represents the transcription termination site. The band at the top of lanes 3 and 4 is the probe reannealed to itself.

TABLE 2. Subcellular distribution of enzymes in *PUT1-lacZ* strains

Strain[a]	Enzyme[b]	% Activity in:	
		Cytosol	Pellet
MB1433(*PUT1-lacZ25*)	G6PDH	96	4
	Cyt *c* oxi	6	94
	β-Gal	89	11
MB1433(*PUT1-lacZ128*)	G6PDH	96	4
	Cyt *c* oxi	9	91
	β-Gal	10	90
MB1433(*PUT1-lacZ455*)	G6PDH	96	4
	Cyt *c* oxi	7	93
	β-Gal	6	94

[a] The *PUT1-lacZ455*, *PUT1-lacZ128*, or *PUT1-lacZ25* gene fusion was carried on plasmid YCp50 in strain MB1433. The number that follows *lacZ* indicates the number of *PUT1* codons in the gene fusion. Growth medium contained 0.5% galactose, 0.2% ammonium sulfate, 0.1% proline, and tryptophan (20 mg/liter).

[b] Abbreviations: G6PDH, glucose-6-phosphate dehydrogenase; Cyt *c* oxi, cytochrome *c* oxidase; β-Gal, β-galactosidase.

different environmental or cellular signals on the production of a cytoplasmic β-galactosidase encoded by the *PUT1-lacZ25* gene fusion. This gene fusion contained approximately 1 kb of upstream sequences and was located on a single-copy plasmid pWB36 (Fig. 1). In an attempt to localize the sequences responsible for the different types of regulation, two promoter deletions were made. Plasmid pWB38 was derived from plasmid pWB36 by deleting about 0.5 kb of DNA 5′ of the *Sau3A* site. Plasmid pWB40 was also derived from plasmid pWB36 and contained only about 300 bp upstream of the *PUT1* gene, 3′ from the *Msp*I site (see Fig. 1 and 2).

Because an intact respiratory chain is required for proline utilization, we examined the *PUT1* gene for regulation by the presence of oxygen, respiratory deficiency, carbon catabolite repression and the cytochrome regulatory system.

(i) Regulation by oxygen. Strain MB1433 carrying each of the three *PUT1-lacZ* plasmids, pWB36 (−1010 to −1 promoter), pWB38 (−458 to −1 promoter), and pWB40 (−293 to −1 promoter), or the control plasmid YCp1Z containing a *CYC1-lacZ* gene fusion, was grown in the presence or absence of oxygen. Table 3 shows that the expression of *PUT1-lacZ* with the complete promoter decreased approximately 10-fold in the absence of oxygen compared with the level found in the presence of oxygen. As expected, the *CYC1-lacZ* control behaved in a similar manner, consistent with the previous report that *CYC1* gene expression is under oxygen regulation (19).

The addition of proline to aerobically grown cells led to the characteristic eightfold increase in expression previously seen with proline oxidase activity (8, 38). Addition of proline to anaerobically grown cells also led to induction of *PUT1-lacZ* expression to an even greater extent than under aerobic conditions. From these data, we conclude that *PUT1* expression is oxygen regulated, as well as proline inducible, and the two seem to be independent.

Expression of β-galactosidase from the plasmid carrying the −458 to −1 promoter was comparable to the wild-type promoter in the absence of proline and was still proline inducible, both aerobically and anaerobically, although to only 75% of the values of the wild-type promoter. This deleted promoter lacks the unusual 9-bp tandem repeat, which suggests that the repeat does not play an important role in regulation by either oxygen or proline.

In contrast, the plasmid carrying the shortest promoter, nucleotides −293 to −1, lost the response to oxygen; the basal level (no proline added) of β-galactosidase activity from anaerobically grown cells was the same as that from aerobically grown cells, approximately sixfold lower than the wild-type promoter. However, expression from this plasmid was still proline inducible, increasing approximately eightfold under aerobic conditions and fivefold under anaerobic conditions.

In cells grown aerobically on a medium containing proline as the sole nitrogen source, β-galactosidase activity increased approximately 40-fold over uninduced levels for the three plasmids (data not shown).

These results suggest that the sequences located between positions −458 and −293 from the translation initiation site are required for enhanced expression in the presence of oxygen and also are important for maximum expression with or without proline. There must be additional sequences that contribute to proline induction closer to the initiation site than the −293 position.

(ii) Respiratory deficiency. Vegetative petite strains of *S. cerevisiae* that are genetically *PUT1* and *PUT2* cannot utilize proline as their sole nitrogen source because of lack of a

TABLE 3. Regulation of *PUT1* gene expression

Strain[a]	Relevant genotype	*PUT1* upstream sequence[b]	Sp act, β-Gal[c]				$+O_2/-O_2$[d]
			$+O_2$		$-O_2$		
			−Pro	+Pro	−Pro	+Pro	
MB1433(pWB36)	[*rho*$^+$]	−1010 to −1	444	3,492	43	1,064	10
MB1433(pWB38)	[*rho*$^+$]	−458 to −1	421	2,307	48	724	9
MB1433(pWB40)	[*rho*$^+$]	−293 to −1	70	559	80	414	1
MB1433(YCp1Z)	[*rho*$^+$]		28	ND	3	ND	9
C1500(pWB36)	[*rho*$^-$]	−1010 to −1	496	5,930	456	4,310	1
BWG1-7A(pWB36)	*HAP1*	−1010 to −1	920	3,904	NG	NG	
WB32(pWB36)	*hap1*	−1010 to −1	881	3,669	NG	NG	
C70-1B(pWB36)	*HAP2*	−1010 to −1	569	4,765	NG	NG	
C70-10A(pWB36)	*hap2*	−1010 to −1	728	3,265	NG	NG	

[a] *PUT1-lacZ* fusion plasmids were introduced into various strains by transformation. Strain C1500 is an ethidium bromide-induced, respiratory-deficient derivative of strain MB1433. Strains C70-1B and C70-10A are meiotic products of the diploid strain C70 described in Materials and Methods.

[b] Sequence position is related to the translational start site (+1) of the *PUT1* gene.

[c] Units of specific activity for β-galactosidase (β-Gal) are nanomoles of *o*-nitrophenol formed per minute per milligram of protein. Each value represents the average of two or more determinations. Abbreviations: $+O_2$, aerobic growth; $-O_2$, anaerobic growth; −pro, without proline; +pro, with proline; ND, not determined; NG, no growth. Growth conditions are described in Materials and Methods.

[d] Ratio of levels of enzyme activity in aerobic conditions/levels of enzyme activity in anaerobic conditions in the absence of proline.

functional electron transport chain. P5C dehydrogenase is measurable in vitro from extracts of [*rho*$^-$] strains, and recent attempts to measure proline oxidase in vitro with artificial electron acceptors in the assay have also been successful (S.-S. Wang and M. C. Brandriss, unpublished results).

We were interested in the effect of respiratory capacity on induction by proline and on the regulation by oxygen. Table 3 shows that the level of β-galactosidase activity in the respiratory-deficient strain C1500 carrying plasmid pWB36 under aerobic conditions was comparable to that in the wild-type strain, and induction by proline was also similar to that of the wild type. The hyperinduction by proline seen in the respiratory-deficient strain is a phenomenon that has been described previously (10).

In contrast, oxygen regulation was completely abolished in the respiratory-deficient strain; aerobic levels of β-galactosidase were similar to anaerobic levels. Proline induction, however, was still present.

(iii) Carbon catabolite repression. We compared the expression of the wild-type *PUT1-lacZ* plasmid with that of the *CYC1-lacZ* plasmid in strain 1433 grown aerobically with 2% glucose or 2% raffinose as the sole carbon source. The relief of carbon catabolite repression had a small (threefold) effect on the β-galactosidase levels measured from *PUT1-lacZ*. (The specific activities of glucose and raffinose were 328 and 994, respectively). In contrast, the *CYC1-lacZ* gene fusion (41) showed a 46-fold derepression. (Specific activities of glucose and raffinose were 18 and 823, respectively).

(iv) Cytochrome regulators *HAP1* and *HAP2*. The *HAP1* and *HAP2* genes have been shown to regulate the expression of *CYC1* and other genes encoding respiratory functions (17, 33). As shown in Table 3, the level of β-galactosidase activity measured in strain WB32(*hap1*) harboring the *PUT1-lacZ* plasmid was comparable to that from strain BWG1-7A(*HAP1*) carrying the same plasmid. Similar results were found in strains carrying either *HAP2* (strain C70-1B) or *hap2* (C70-10A). The absolute levels of β-galactosidase activity cannot be directly compared to those of MB1433, since these strains are not closely related. These results indicate that the *PUT1* gene does not respond to the global regulators of the cytochrome system.

DISCUSSION

The proline utilization pathway in *S. cerevisiae* was first characterized by the study of mutations affecting the ability of strains to use proline as the sole source of nitrogen (8). The *put1* mutations result in deficiencies in proline oxidase activity that show a gene dosage effect in heterozygotes (9). The *PUT1* gene was cloned by functional complementation of a *put1* mutant of *S. cerevisiae* (38). The cloned gene was used to show that the increase in steady-state levels of mRNA upon addition of proline correlated well with increased expression of enzyme activity. The *PUT1* message and *PUT1-lacZ* gene expression respond to the effects of mutations in the *PUT3* gene (6a, 38) in the same manner as does proline oxidase activity (9). In this report, we demonstrate that the *PUT1* gene product is a mitochondrially imported protein. Although the definitive proof that the *PUT1* gene encodes proline oxidase is still lacking, we are confident that *PUT1* is the structural gene for this enzyme.

Proline oxidase carries out the first step in the conversion of proline to glutamate. This enzyme was shown to be associated with the inner mitochondrial membrane in rat liver (12) and with the plasma membrane in *E. coli* (36) and *Salmonella typhimurium* (27). In *S. cerevisiae*, its requirement for a functional electron transport chain has previously been demonstrated (8, 10, 28), as has the mitochondrial location of the second enzyme in the pathway, Δ^1-pyrroline-5-carboxylate dehydrogenase (7, 11). The hydropathy plot shown in Fig. 3 indicates that the *PUT1* gene product is not an integral membrane protein but may have a membrane anchor in residues 75 to 112. In our recent attempts to measure proline oxidase activity, we have found that the enzyme, like the *PUT1-lacZ* hybrid protein, fractionates with mitochondria but is loosely associated (S.-S. Wang and M. C. Brandriss, unpublished results). This finding is similar to that reported by Menzel and Roth for the *Salmonella typhimurium* enzyme (27). To date, no protein sequence data on proline oxidase from any organism has been reported.

The *lacZ* gene fusions enabled us to demonstrate that the 125 amino-terminal residues of the *PUT1* gene product have sufficient information to target β-galactosidase to mitochondria. The *PUT1* and *PUT2* signal sequences do not resemble each other in primary sequence but do share characteristics common to mitochondrially imported proteins, namely, a net positive charge and many hydroxylated amino acids (22; for a review, see reference 15).

Analysis of the *PUT1* DNA sequence revealed a TATA box at −118 from the initiation of translation and 72 bp upstream from the major transcription start sites. The *PUT1* transcription initiation sites occur in a cluster that spans approximately eight nucleotides. This contrasts strikingly with the *PUT2* gene in which transcription begins at 12 to 14 different sites scattered over 50 bp (22). On the basis of steady-state mRNA levels and β-galactosidase levels (measured from either *PUT1-lacZ* or *PUT2-lacZ* gene fusions), the genes are expressed at about the same level in uninducing conditions but differ in their level of expression under partially or fully inducing conditions. The induction ratio (expression on proline as the nitrogen source/expression on ammonia as the nitrogen source) for the *PUT1* gene is approximately 50:1, while that of *PUT2* is 10:1 to 15:1.

From the behavior of the promoter deletion mutations, it appears that the region between −458 and −293 is important for full expression of the *PUT1* gene under both noninducing and inducing conditions. A preliminary comparison between the *PUT1* and *PUT2* upstream regions has indicated that there are several homologous sequences present, and experiments are in progress to determine which, if any, have significance for the coregulation of these genes (A. H. Siddiqui and M. C. Brandriss, unpublished results).

The same upstream sequence (−458 to −293) appears to contain an element responsible for oxygen regulation. No significant homology was found between sequences in this region and the positive and negative elements involved in the oxygen regulation of the yeast *CYC7* gene (41). Although this 160-bp region is important for the full expression of the *PUT1* gene, as well as for oxygen regulation, there must be additional *cis*-acting elements independently controlling proline induction of *PUT1* gene expression downstream of position −293.

Of the environmental or cellular signals that we tested for effect on proline utilization, only proline and oxygen appeared to influence gene expression. In spite of the importance of a functional electron transport chain to the activity of proline oxidase, *PUT1* did not respond to elements that are known to affect the cytochrome system, such as carbon catabolite repression, respiratory deficiency, and the HAP proteins. The *PUT1* gene resembles the *CYC1* (iso-1-cytochrome *c*) gene in that it is transcribed in respiratory-

deficient strains (32) and is also under oxygen regulation (41). However, in the case of *PUT1*, heme apparently is not involved in its regulation. It is possible that, like its counterpart in the enteric bacteria (27), the *S. cerevisiae* proline oxidase is a flavoprotein.

A surprising finding was that oxygen regulation was abolished in a [*rho*$^-$] strain grown anaerobically, i.e., the *PUT1* gene is expressed constitutively. Genetically speaking, we could say that under anaerobic conditions, this petite regulation is epistatic to oxygen regulation. A recent study by Parikh et al. (32) concerns the existence of such petite regulation. They demonstrated that the state of the mitochondrial genome could alter the expression of certain nuclear genes in *S. cerevisiae*. Using aerobic growth conditions, these workers reported that certain nuclear genes were hyperexpressed in various mit$^-$, [*rho*$^-$], and [*rho*^{0}] strains, compared with their wild-type [*rho*$^+$] parent. The behavior of the *PUT1* gene under anaerobic conditions resembles that seen by Parikh et al. in the [*rho*$^-$] strain; *PUT1* expression increased 10-fold compared to its [*rho*$^+$] parent. This effect is not seen under aerobic conditions, perhaps because maximum gene expression has already been achieved or because other regulatory phenomena exist under aerobic conditions to obscure it.

Since the *PUT1* gene product requires a functional electron transport chain for its enzymatic activity, we find it paradoxical that the *PUT1* gene appears to be expressed under conditions in which some or all members of the electron transport chain are nonfunctional. In respiratory-deficient strains, in severely carbon-repressed conditions, and in a *hap2* mutant, *PUT1* (as measured by *PUT1-lacZ* expression) is regulated at wild-type levels. Even under anaerobic conditions, the *PUT1* gene product, although reduced 10-fold, is measurable. The production of this enzymatically inactive protein appears to be a waste of cellular energy unless the enzyme serves another function. At this time, we cannot rule out this intriguing possibility.

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